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APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

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 - Statement Regarding Fed sponsored R&D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings *(if filed)*
 - Detailed Description of the Invention *(including drawings, if filed)*
 - Claim(s)
 - Abstract of the Disclosure
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The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

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**RECOMBINANT PARAINFLUENZA VIRUS
EXPRESSION SYSTEMS AND VACCINES**

5

1. INTRODUCTION

10 The present invention relates to recombinant parainfluenza virus (PIV) cDNA or RNA which may be used to express heterologous gene products in appropriate host cell systems and/or to rescue negative strand RNA recombinant viruses that express, package, and/or present the heterologous gene product. The chimeric viruses and expression products may advantageously be used in vaccine formulations including vaccines against a broad range of pathogens and antigens. The present invention relates to chimeric viruses comprising human PIV or bovine PIV genomic sequences and nucleotide sequences encoding heterologous antigens. In particular, the present invention encompasses vaccine
15 preparations comprising chimeric PIV expressing antigenic glycoproteins of another species of PIV or of another virus. In one embodiment, the present invention relates to a cross-species bPIV3/hPIV3 that is viable and infectious.

20 The present invention also relates to genetically engineered parainfluenza viruses that contain modifications and/or mutations that make the recombinant virus suitable for use in vaccine formulations, such as an attenuated phenotype or enhanced immunogenicity. The present invention relates to the use of the recombinant parainfluenza viruses and viral vectors against a broad range of pathogens and/or antigens, including tumor specific antigens. The invention is demonstrated by way of examples in which recombinant parainfluenza virus cDNA or RNA was constructed containing heterologous gene coding
25 sequences in the positive or negative polarity which were then used to rescue the negative strand RNA chimeric virus particles and/or to express the heterologous gene products which may then be utilized in vaccine preparations. In particular, such heterologous gene sequences include sequences derived from another species of PIV.

30

2. BACKGROUND OF THE INVENTION

5 Parainfluenza viral infection results in serious respiratory tract disease in infants and children. (Tao, et al., 1999, Vaccine 17: 1100-08). Infectious parainfluenza viral infections account for approximately 20% of all hospitalizations of pediatric patients suffering from respiratory tract infections worldwide. *Id.* A vaccine has not yet been approved for the prevention of PIV related disease, nor is there an effective antiviral therapy once disease occurs.

10 PIV is a member of the paramyxovirus genus of the paramyxovirus family. PIV is made up of two structural modules: (1) an internal ribonucleoprotein core, or nucleocapsid, containing the viral genome, and (2) an outer, roughly spherical lipoprotein envelope. Its genome is a single strand of negative sense RNA, approximately 15,456 nucleotides in length, encoding at least eight polypeptides. These proteins include the nucleocapsid structural protein (NP, NC, or N depending on the genera), the phosphoprotein (P), the matrix protein (M), the fusion glycoprotein (F), the hemagglutinin-neuraminidase glycoprotein (HN), the large polymerase protein (L), and the C and D proteins of unknown function. *Id.*

15 The parainfluenza nucleocapsid protein (NP, NC, or N) consists of two domains within each protein unit including an amino-terminal domain, comprising about two-thirds of the molecule, which interacts directly with the RNA, and a carboxyl-terminal domain, which lies on the surface of the assembled nucleocapsid. A hinge is thought to exist at the junction of these two domains thereby imparting some flexibility to this protein (see Fields *et al.* (ed.), 1991, Fundamental Virology, Second Edition, Raven Press, New York, incorporated by reference herein in its entirety). The matrix protein (M), is apparently involved with viral assembly and interacts with both the viral membrane as well as the nucleocapsid proteins. The phosphoprotein (P), which is subject to phosphorylation, is thought to play a regulatory role in transcription, and may also be involved in methylation, phosphorylation and polyadenylation. The fusion glycoprotein (F) interacts with the viral membrane and is first produced as an inactive precursor, then cleaved post-translationally to produce two disulfide linked polypeptides. The active F protein is also involved in penetration of the parainfluenza virion into host cells by facilitating fusion of the viral envelope with the host cell plasma membrane. *Id.* The glycoprotein, hemagglutinin-

neuraminidase (HN), protrudes from the envelope allowing the virus to contain both hemagglutinin and neuraminidase activities. HN is strongly hydrophobic at its amino terminal which functions to anchor the HN protein into the lipid bilayer. *Id.* Finally, the large polymerase protein (L) plays an important role in both transcription and replication. *Id.*

In one embodiment, the present invention relates to the construction of a cross-species bovine PIV3/human PIV3 chimeric virus vaccine. Bovine parainfluenza virus was first isolated in 1959 from calves showing signs of shipping fever. It has since been isolated from normal cattle, aborted fetuses, and cattle exhibiting signs of respiratory disease (Breker-Klassen, et al., 1996, Can. J. Vet. Res. 60: 228-236). *See also* Shibuta, 1977, Microbiol. Immunol. 23 (7), 617-628. Human and bovine PIV3 share neutralizing epitopes but show distinct antigenic properties. Significant differences exist between the human and bovine viral strains in the HN protein. In fact, while a bovine strain induces some neutralizing antibodies to hPIV infection, a human strain seems to induce a wider spectrum of neutralizing antibodies against human PIV3 (Klippmark, et al., 1990, J. Gen. Vir. 71: 1577-1580). Thus, it is expected that the bPIV3/hPIV3 chimeric virus vaccine of the present invention will also induce a wider spectrum of neutralizing antibodies against hPIV3 infection while remaining attenuated and safe for human use. Other chimeric parainfluenza virus vaccines are also contemplated by the invention.

The replication of all negative-strand RNA viruses, including PIV, is complicated by the absence of cellular machinery required to replicate RNA. Additionally, the negative-strand genome can not be translated directly into protein, but must first be transcribed into a positive-strand (mRNA) copy. Therefore, upon entry into a host cell, the genomic RNA alone cannot synthesize the required RNA-dependent RNA polymerase. The L, P and N proteins must enter the cell along with the genome on infection.

It is hypothesized that most or all of the viral proteins that transcribe PIV mRNA also carry out their replication. The mechanism that regulates the alternative uses (i.e., transcription or replication) of the same complement of proteins has not been clearly identified but appears to involve the abundance of free forms of one or more of the nucleocapsid proteins. Directly following penetration of the virus, transcription is initiated

by the L protein using the negative-sense RNA in the nucleocapsid as a template. Viral RNA synthesis is regulated such that it produces monocistronic mRNAs during transcription.

5 Following transcription, virus genome replication is the second essential event in infection by negative-strand RNA viruses. As with other negative-strand RNA viruses, virus genome replication in PIV is mediated by virus-specified proteins. The first products of replicative RNA synthesis are complementary copies (*i.e.*, plus-polarity) of PIV genome RNA (cRNA). These plus-stranded copies (anti-genomes) differ from the plus-strand
10 mRNA transcripts in the structure of their termini. Unlike the mRNA transcripts, the anti-genomic cRNAs are not capped and methylated at the 5' termini, and are not truncated and polyadenylated at the 3' termini. The cRNAs are coterminal with their negative strand templates and contain all the genetic information in the complementary form. The cRNAs serve as templates for the synthesis of PIV negative-strand viral genomes (vRNAs).

15 Both the bPIV negative strand genomes (vRNAs) and antigenomes (cRNAs) are encapsidated by nucleocapsid proteins; the only unencapsidated RNA species are virus mRNAs. For bPIV, the cytoplasm is the site of virus RNA replication, just as it is the site for transcription. Assembly of the viral components appears to take place at the host cell plasma membrane and mature virus is released by budding.

20

2.1. ENGINEERING NEGATIVE STRAND RNA VIRUSES

The RNA-directed RNA polymerases of animal viruses have been extensively studied with regard to many aspects of protein structure and reaction conditions. However, the elements of the template RNA which promote optimal expression by the polymerase
25 could only be studied by inference using existing viral RNA sequences. This promoter analysis is of interest since it is unknown how a viral polymerase recognizes specific viral RNAs from among the many host-encoded RNAs found in an infected cell.

Animal viruses containing plus-sense genome RNA can be replicated when plasmid-derived RNA is introduced into cells by transfection (for example, Racaniello et al., 1981,
30 Science 214:916-919; Levis, et al., 1986, Cell 44: 137-145). In the case of poliovirus, the purified polymerase will replicate a genome RNA in *in vitro* reactions and when this plus-

sense RNA preparation is transfected into cells it is infectious (Kaplan, et al., 1985, Proc. Natl. Acad. Sci. USA 82:8424-8428). However, the template elements which serve as transcription promoter for the poliovirus-encoded polymerase are unknown since even RNA
5 homopolymers can be copied (Ward, et al., 1988, J. Virol. 62: 558-562). SP6 transcripts have also been used to produce model defective interfering (DI) RNAs for the Sindbis viral genome. When the RNA is introduced into infected cells, it is replicated and packaged. The RNA sequences which were responsible for both recognition by the Sindbis viral
10 polymerase and packaging of the genome into virus particles were shown to be within 162 nucleotides (nt) of the 5' terminus and 19 nt of the 3' terminus of the genome (Levis, et al., 1986, Cell 44: 137-145). In the case of brome mosaic virus (BMV), a positive strand RNA plant virus, SP6 transcripts have been used to identify the promoter as a 134 nt tRNA-like 3' terminus (Dreher, and Hall, 1988, J. Mol. Biol. 201: 31-40). Polymerase recognition and
15 synthesis were shown to be dependent on both sequence and secondary structural features (Dreher, et al., 1984, Nature 311: 171-175).

The negative-sense RNA viruses have been refractory to study with respect to the sequence requirements of the replicase. The purified polymerase of vesicular stomatitis virus is only active in transcription when virus-derived ribonucleoprotein complexes (RNPs)
20 are included as template (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 126: 40-49; Emerson and Yu, 1975, J. Virol. 15: 1348-1356; Naito and Ishihama, 1976, J. Biol. Chem. 251: 4307-4314). With regard to influenza viruses, it was reported that naked RNA purified from virus was used to reconstitute RNPs. The viral nucleocapsid and polymerase proteins were gel-purified and renatured on the viral RNA using thioredoxin (Szewczyk, et
25 al., 1988, Proc. Natl. Acad. Sci. USA, 85: 7907-7911). However, these authors did not show that the activity of the preparation was specific for influenza viral RNA, nor did they analyze the signals which promote transcription.

It is now possible to recover negative strand RNA viruses using a recombinant reverse genetics approach. See U.S. Patent No. 5,166,057 to Palese et al., incorporated
30 herein by reference in its entirety. Although this method was originally applied to engineer influenza viral genomes (Luytjes et al. 1989, Cell 59: 1107-1113; Enami et al. 1990, Proc. Natl. Acad. Sci. USA 92: 11563-11567), it has been successfully applied to a wide variety of

segmented and nonsegmented negative strand RNA viruses, including rabies (Schnell et al. 1994, EMBO J. 13:4195-4203); respiratory syncytial virus (Collins et al. 1991, Proc. Natl. Acad. Sci. USA 88:9663-9667); and Sendai virus (Park et al. 1991, Proc. Natl. Acad. Sci. USA 88:5537-5541; Kato et al., 1996, Genes Cells 1:569-579).

The reverse genetics has been successfully applied to rescue other minigenomes of PIV3, i.e., cDNAs that encode vRNA in which all the viral genes were replaced by a negative-sense copy of the CAT gene (Dimock et al., 1993, J. Virol. 67: 2772-2778). In this study, reverse genetics was employed to identify the minimum PIV3 3' terminal and 5' terminal nucleotide sequences required for replication, gene expression and transmission of PIV. An infectious human PIV3 was rescued when the reverse genetics approach was successfully applied to recover virus from cells transfected with cDNAs, separately encoding a complete hPIV3 genome, hPIV3 nucleocapsid protein N, the phosphoprotein P and polymerase protein L (Durbin & Banerjee, 1997, J. Virol. 235:323-332).

The reverse genetics approach has also been applied to engineer recombinant parainfluenza genomes for the production of recombinant human PIV for the purpose of generating vaccines. See WO 98/53078, entitled "Production of Attenuated Parainfluenza Virus Vaccines From Cloned Nucleotide Sequences," by Murphy et al. However, the approach has never been heretofore applied to successfully engineer a PIV3 containing heterologous sequences which has suitable properties for use in vaccines to be administered to humans.

3. SUMMARY OF THE INVENTION

Recombinant parainfluenza virus cDNA and RNA is described which may be used with expression plasmids and/or helper virus to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. In one embodiment, the present invention relates to engineering recombinant bovine or human parainfluenza viruses which express heterologous antigens. In particular, the invention relates to engineering a recombinant Kansas-strain bovine parainfluenza type 3 virus containing heterologous sequences as well as cDNA and RNA molecules coding for the same. The present invention also relates to recombinant PIV which contain modifications

which result in phenotypes which make the chimeric virus more suitable for use in vaccine formulations, and which contain heterologous genes, including genes of other species of PIV, other viruses, pathogens, cellular genes, tumor antigens, etc.

5 The present invention relates to vaccines comprising the chimeric viruses and vectors described herein. The present invention also relates to vaccine formulations suitable for administration to humans, as well as veterinary uses. For example, the vaccines of the present invention may be designed for administration to humans, including children, domestic animals, including cats and dogs; wild animals, including foxes and racoons;
10 livestock and fowl, including horses, cattle, sheep, turkeys and chickens.

 In another embodiment, the present invention relates to engineering recombinant parainfluenza viruses and viral vectors which encode combinations of genes from different strains of PIV or which contain heterologous genes including genes of other viruses, pathogens, cellular genes, tumor antigens, etc. Thus, the invention encompasses
15 recombinant parainfluenza vectors and viruses which are engineered to encode genes from different species and strains of the parainfluenza virus, including the F and HN genes of human PIV3.

 In a further embodiment, rescue of the chimeric virus or expression products may be achieved by reverse genetics in host cell systems where the host cells are transfected with
20 chimeric cDNA or RNA constructs. The RNA templates of the present are prepared by transcription of appropriate DNA sequences with a DNA-directed RNA polymerase. The resulting RNA templates are of the negative-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template. Alternatively, positive-polarity RNA templates which contain appropriate terminal
25 sequences which enable the viral RNA-synthesizing apparatus to recognize the template, may also be used. Expression from positive polarity RNA templates may be achieved by transfection of plasmids having promoters which are recognized by the DNA-dependent RNA polymerase. For example, plasmid DNA encoding positive RNA templates under the control of a T7 promoter can be used in combination with the vaccinia virus T7 system.

30 Bicistronic mRNAs can be constructed to permit internal initiation of translation of viral sequences and allow for the expression of foreign protein coding sequences from the

regular terminal initiation site, or vice versa. Alternatively, a foreign protein may be expressed from internal transcriptional unit in which the transcriptional unit has an initiation site and polyadenylation site. In another embodiment, the foreign gene is inserted into a PIV gene such that the resulting expressed protein is a fusion protein.

The recombinant mutant parainfluenza viral cDNA and RNA templates of the present invention may be used to transfect transformed cell lines that express the RNA dependent RNA-polymerase and allow for complementation. Alternatively, a plasmid expressing from an appropriate promoter, can be used for virus specific (chimeric) RNA transfection. Complementation may also be achieved with the use of a helper virus which provides the RNA dependent RNA-polymerase. Additionally, a non-virus dependent replication system for parainfluenza virus is also described. The minimum subset of parainfluenza virus proteins needed for specific replication and expression of the virus are the three proteins, L, P, and N or NP, which can be expressed from plasmids by a vaccinia virus T7 or other system. When plasmids encoding an antigenomic copy of the PIV genome are used to supply the viral genome, the minimum subset of virus proteins that may be needed for specific replication and expression of the virus are the L and P proteins, since when the antigenomic copy of the genome is transcribed, the N or NP polymerase protein is the first protein transcribed, thus it is not necessary to additionally provide the N or NP polymerase in trans.

The expression products and/or chimeric virions obtained may advantageously be utilized in vaccine formulations. The expression products and chimeric virions of the present invention may be engineered to create vaccines against a broad range of pathogens, including viral and bacterial antigens, tumor antigens, allergen antigens, and auto antigens involved in autoimmune disorders. In particular, the chimeric virions of the present invention may be engineered to create anti- human parainfluenza vaccines, wherein the bovine parainfluenza fusion (F) and hemagglutinin (HN) glycoproteins are replaced by the human F and HN glycoproteins to construct a chimeric bPIV/hPIV vaccine for use in humans. In another embodiment, the chimeric virions of the present invention may be engineered to create anti-HIV vaccines, wherein an immunogenic polypeptide from gp160, and/or from internal proteins of HIV is engineered into the glycoprotein HN protein to

construct a vaccine that is able to elicit both vertebrate humoral and cell-mediated immune responses. In yet another embodiment, the invention relates to recombinant parainfluenza viral vectors and viruses which are engineered to encode mutant parainfluenza viral genes or to encode combinations of genes from different strains of parainfluenza virus.

3.1. DEFINITIONS

As used herein, the following terms will have the meanings indicated:

10	cDNA =	complementary DNA
	L =	large protein
	M =	matrix protein (lines inside of envelope)
	F =	fusion glycoprotein
	HN =	hemagglutinin-neuraminidase glycoprotein
	N, NP or NC =	nucleoprotein (associated with RNA and required for polymerase activity)
15	P =	phosphoprotein
	MOI =	multiplicity of infection
	NA =	neuraminidase (envelope glycoprotein)
	PIV =	parainfluenza virus
	bPIV =	bovine parainfluenza virus
	hPIV =	human parainfluenza virus
	bPIV/hPIV =	recombinant bPIV with hPIV sequences
20	nt =	nucleotide
	RNP =	ribonucleoprotein
	rRNP =	recombinant RNP
	vRNA =	genomic virus RNA
	cRNA =	antigenomic virus RNA

4. DESCRIPTION OF FIGURES

Figure 1. PCR fragments from nt 5255 to nt 6255 derived from three different isolates of the bPIV3/hPIV3 chimeric virus were amplified. The resulting 1 kb DNA fragments were digested with enzymes specific for the F gene of human PIV3. These enzymes do not cut in the corresponding fragment of bovine PIV3. The 1% agarose gel shows the undigested fragment (lanes 2,5, and 6) and the SacI or BgIII digested fragments (lanes 4, 6 and lanes 9, 10, and 11, respectively). The sample in lane 10 is undigested,

however, upon a repeat of digestion with BgIII, this sample was cut (data not shown). Lanes 1 and 8 show a DNA size marker.

5 Figure 2. PCR fragments from nt 9075 to nt 10469 derived from three different
isolates of the bPIV3/hPIV3 chimeric virus were amplified. The resulting 1.4kb DNA
fragments were digested with enzymes specific for the L gene of bovine PIV3. These
enzymes do not cut in the corresponding fragment of human PIV3. The 1% agarose gel
shows the undigested 1.4 kb fragment (lanes 2, 5, and 8). The smaller DNA fragments
10 produced by digestion with BamH1 and PvuII are shown in lanes 3, 4, 6, 7, 9, and 10). Lane
1 shows a DNA size marker.

5. DESCRIPTION OF THE INVENTION

15 The present invention relates to recombinant parainfluenza cDNA and RNA
constructs including, more specifically, recombinant Kansas strain bovine PIV3 cDNA and
RNA constructs, which may be used to express heterologous gene products in appropriate
host cell systems and/or to rescue negative strand RNA recombinant viruses that express,
package, and/or present the heterologous gene product. These chimeric viruses and
expression products may be used as vaccines suitable for administration to humans or
20 animals. In a specific embodiment of the invention, the heterologous gene product is a
peptide or protein derived from the genome of a human immunodeficiency virus.

In one embodiment the invention relates to PIV cDNA constructs derived from
human or bovine PIV variants, which may be used to express heterologous genes encoding
foreign antigens and other products from a variety of pathogens, cellular genes, tumor
25 antigens, and viruses including genes encoding glycoproteins of different species of virus.
Thus, the invention encompasses recombinant parainfluenza vectors and viruses which are
engineered to encode genes from different species or strains of the parainfluenza virus as
well as foreign antigens from pathogens, tumors, allergens, and auto antigens involved in
autoimmune disorders. The invention also relates to recombinant parainfluenza viral
30 vectors and viruses which are engineered to encode mutant parainfluenza sequences of the
same species or strain.

In a further embodiment, rescue of the chimeric virus or expression products may be achieved by reverse genetics in host cell systems where the host cells are transfected with chimeric cDNA or RNA constructs. The RNA templates of the present are prepared by transcription of appropriate DNA sequences with a DNA-directed RNA polymerase. The RNA templates of the present invention may be prepared either in vitro or in vivo by transcription of appropriate DNA sequences using a DNA-directed RNA polymerase such as bacteriophage T7, T3, the SP6 polymerase or a eukaryotic polymerase such as polymerase I. The resulting RNA templates are of the negative-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template. Alternatively, positive-polarity RNA templates which contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template, may also be used. Expression from positive polarity RNA templates may be achieved by transfection of plasmids having promoters which are recognized by the DNA-dependent RNA polymerase. For example, plasmid DNA encoding positive RNA templates under the control of a T7 promoter can be used in combination with the vaccinia virus T7 system.

Bicistronic mRNAs can be constructed to permit internal initiation of translation of viral sequences and allow for the expression of foreign protein coding sequences from the regular terminal initiation site, or vice versa. Alternatively, a foreign protein may be expressed from internal transcriptional unit in which the transcriptional unit has an initiation site and polyadenylation site. In another embodiment, the foreign gene is inserted into a PIV gene such that the resulting expressed protein is a fusion protein.

The recombinant mutant parainfluenza viral cDNA and RNA templates of the present invention may be used to transfect transformed cell lines that express the RNA dependent RNA-polymerase and allow for complementation. For example, the recombinant RNA templates may be used to transfect continuous/transfected cell lines that express the RNA-directed RNA polymerase proteins allowing for complementation. In a preferred embodiment, a non-virus dependent replication system is used to recover chimeric PIV, in which plasmid DNA encoding the PIV genome or antigenome is coexpressed with plasmid DNA encoding the minimum subset of PIV proteins needed for specific replication and expression of the virus, as demonstrated by way of working example as described *infra*.

Alternatively, a plasmid expressing from an appropriate promoter, can be used for virus specific (chimeric) cDNA or RNA transfection. Complementation may also be achieved with the use of a helper virus which provides the RNA dependent RNA-
5 polymerase. Additionally, a non-virus dependent replication system for parainfluenza virus is also described. The minimum subset of parainfluenza virus proteins needed for specific replication and expression of the virus are the three proteins, L, P, and N or NP, which can be expressed from plasmids by a vaccinia virus T7 or other system.

10 The expression products and/or chimeric virions obtained may advantageously be utilized in vaccine formulations. The expression products and chimeric virions of the present invention may be engineered to create vaccines against a broad range of pathogens, including viral and bacterial antigens, tumor antigens, allergen antigens, and auto antigens involved in autoimmune disorders. In particular, the chimeric virions of the present
15 invention may be engineered to create anti- human parainfluenza vaccines, wherein the bovine parainfluenza fusion (F) and hemagglutinin (HN) glycoproteins are replaced by the human F and HN glycoproteins to construct a chimeric bPIV/hPIV vaccine for use in humans. In another embodiment, the chimeric virions of the present invention may be engineered to create anti-HIV vaccines, wherein an immunogenic polypeptide from gp160, and/or from internal proteins of HIV is engineered into the glycoprotein HN protein to
20 construct a vaccine that is able to elicit both vertebrate humoral and cell-mediated immune responses. In yet another embodiment, the invention relates to recombinant parainfluenza viral vectors and viruses which are engineered to encode mutant parainfluenza viral genes or to encode combinations of genes from different strains of parainfluenza virus.

25 In a broader embodiment, the expression products and chimeric virions of the present invention may be engineered to create vaccines against a broad range of pathogens, including viral antigens, tumor antigens and auto antigens involved in autoimmune disorders. One way to achieve this goal involves modifying existing PIV genes to contain foreign sequences in their respective external domains. Where the heterologous sequences are epitopes or antigens of pathogens, these chimeric viruses may be used to induce a
30 protective immune response against the disease agent from which these determinants are derived.

Thus, the present invention relates to the use of viral vectors and chimeric viruses to formulate vaccines against a broad range of viruses and/or antigens including tumor antigens. The viral vectors and chimeric viruses of the present invention may be used to modulate a subject's immune system by stimulating a humoral immune response, a cellular immune response or by stimulating tolerance to an antigen. As used herein, a subject means: humans, primates, horses, cows, sheep, pigs, goats, dogs, cats, avian species and rodents. When delivering tumor antigens, the invention may be used to treat subjects having disease amenable to immunity mediated rejection, such as non-solid tumors or solid tumors of small size. It is also contemplated that delivery of tumor antigens by the viral vectors and chimeric viruses described herein will be useful for treatment subsequent to removal of large solid tumors. The invention may also be used to treat subjects who are suspected of having cancer.

The invention may be divided into the following stages solely for the purpose of description and not by way of limitation: (a) construction of recombinant cDNA and RNA templates; (b) expression of heterologous gene products using recombinant cDNA and RNA templates; and (c) rescue of the heterologous gene in recombinant virus particles.

5.1. CONSTRUCTION OF THE RECOMBINANT cDNA AND RNA

A specific embodiment of the present invention is a chimeric virus comprising a backbone encoded by nucleotide sequences derived from bovine parainfluenza virus genome, such as the Kansas strain of bPIV3, in which bovine parainfluenza virus nucleotide sequences have been substituted with heterologous sequences or in which heterologous sequences have been added to the complete bPIV genome. The present invention also encompasses chimeric viruses comprising a backbone encoded by nucleotide sequences derived from a bPIV genome containing mutations or modifications, in addition to heterologous sequences, which result in a chimeric virus having a phenotype more suitable for use in vaccine formulations, e.g., attenuated phenotype or enhanced antigenicity. In accordance with this particular embodiment of the invention, a heterologous sequence in the context of a bovine PIV3 backbone may be any sequence heterologous to bPIV3.

Another specific embodiment of the present invention is a chimeric virus comprising a backbone encoded by nucleotide sequences derived from human PIV 1, 2, or 3 in which hPIV nucleotide sequences have been substituted with heterologous sequences or in which
5 heterologous sequences have been added to the complete hPIV genome, with the proviso that the resulting chimeric virus is not a chimeric hPIV3 in which the hemagglutinin-neuraminidase and fusion glycoproteins have been replaced by those of hPIV1. The present invention also encompasses chimeric viruses comprising a backbone encoded by nucleotide
10 sequences derived from a hPIV genome containing mutations or modifications, in addition to heterologous sequences, which result in a chimeric virus having a phenotype more suitable for use in vaccine formulations, e.g., attenuated phenotype or enhanced antigenicity.

Heterologous gene coding sequences flanked by the complement of the viral polymerase binding site/promoter, e.g., the complement of 3'-PIV virus terminus of the present invention, or the complements of both the 3'- and 5'-PIV virus termini may be
15 constructed using techniques known in the art. The resulting RNA templates may be of the negative-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template. Alternatively, positive-polarity RNA templates which contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template, may also be used. Recombinant DNA
20 molecules containing these hybrid sequences can be cloned and transcribed by a DNA-directed RNA polymerase, such as bacteriophage T7, T3, the SP6 polymerase or eukaryotic polymerase such as polymerase I and the like, to produce in vitro or in vivo the recombinant RNA templates which possess the appropriate viral sequences that allow for viral
25 polymerase recognition and activity.

In accordance with the present invention, heterologous sequences which may be constructed into the chimeric viruses of the invention include: 1) antigens that are characteristic of a pathogen such as bacteria and viruses; 2) antigens that are characteristic of autoimmune disease; 3) antigens that are characteristic of an allergen; and 4) antigens that are characteristic of a tumor.
30

In a preferred embodiment, heterologous gene sequences that can be expressed into the chimeric viruses of the invention include but are not limited to antigenic epitopes and

glycoproteins of viruses which result in respiratory disease, such as influenza glycoproteins, in particular hemagglutinin H5, H7, respiratory syncytial virus epitopes, New Castle Disease virus epitopes, Sendai virus and infectious Laryngotracheitis virus (ILV). In yet another
5 embodiment of the invention, heterologous gene sequences that can be engineered into the chimeric viruses of the invention include, but are not limited to, viral epitopes and glycoproteins of viruses, such as hepatitis B virus surface antigen, hepatitis A or C virus surface glycoproteins of Epstein Barr virus, glycoproteins of human papilloma virus, simian
10 virus 5 or mumps virus, glycoproteins of herpes virus, VPI of poliovirus, and sequences derived from a human immunodeficiency virus (HIV), preferably type 1 or type 2. In yet another embodiment, heterologous gene sequences that can be engineered into chimeric viruses of the invention include, but are not limited to, Marek's Disease virus (MDV) epitopes, epitopes of infectious Bursal Disease virus (IBDV), epitopes of Chicken Anemia virus, infectious laryngotracheitis virus (ILV), Avian Influenza virus (AIV), rabies, feline
15 leukemia virus, canine distemper virus, vesicular stomatitis virus, and swinepox virus (see Fields *et al.* (ed.), 1991, Fundamental Virology, Second Edition, Raven Press, New York, incorporated by reference herein in its entirety).

Other heterologous sequences of the present invention include antigens that are characteristic of autoimmune disease. These antigens will typically be derived from the cell
20 surface, cytoplasm, nucleus, mitochondria and the like of mammalian tissues, including antigens characteristic of diabetes mellitus, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, pernicious anemia, Addison's disease, scleroderma, autoimmune atrophic gastritis, juvenile diabetes, and discoid lupus erythematosus.

Antigens that are allergens generally include proteins or glycoproteins, including
25 antigens derived from pollens, dust, molds, spores, dander, insects and foods. In addition, antigens that are characteristic of tumor antigens typically will be derived from the cell surface, cytoplasm, nucleus, organelles and the like of cells of tumor tissue. Examples include antigens characteristic of tumor proteins, including proteins encoded by mutated oncogenes; viral proteins associated with tumors; and glycoproteins. Tumors include, but
30 are not limited to, those derived from the types of cancer: lip, nasopharynx, pharynx and oral cavity, esophagus, stomach, colon, rectum, liver, gall bladder, pancreas, larynx, lung

and bronchus, melanoma of skin, breast, cervix, uterine, ovary, bladder, kidney, uterus, brain and other parts of the nervous system, thyroid, prostate, testes, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma and leukemia.

5 In one specific embodiment of the invention, the heterologous sequences are derived from the genome of human immunodeficiency virus (HIV), preferably human immunodeficiency virus-1 or human immunodeficiency virus-2. In another embodiment of the invention, the heterologous coding sequences may be inserted within a PIV gene coding sequence such that a chimeric gene product is expressed which contains the heterologous
10 peptide sequence within the PIV viral protein. In such an embodiment of the invention, the heterologous sequences may also be derived from the genome of a human immunodeficiency virus, preferably of human immunodeficiency virus-1 or human immunodeficiency virus-2.

15 In instances whereby the heterologous sequences are HIV-derived, such sequences may include, but are not limited to sequences derived from the env gene (i.e., sequences encoding all or part of gp160, gp120, and/or gp41), the pol gene (i.e., sequences encoding all or part of reverse transcriptase, endonuclease, protease, and/or integrase), the gag gene (i.e., sequences encoding all or part of p7, p6, p55, p17/18, p24/25) tat, rev, nef, vif, vpu, vpr, and/or vpx.

20 In yet another embodiment, heterologous gene sequences that can be engineered into the chimeric viruses include those that encode proteins with immunopotentiating activities. Examples of immunopotentiating proteins include, but are not limited to, cytokines, interferon type 1, gamma interferon, colony stimulating factors, and interleukin -1, -2, -4, -5, -6, -12.

25 In addition, other heterologous gene sequences that may be engineered into the chimeric viruses include antigens derived from bacteria such as bacterial surface glycoproteins, antigens derived from fungi, and antigens derived from a variety of other pathogens and parasites. Examples of heterologous gene sequences derived from bacterial pathogens include, but are not limited to, antigens derived from species of the following
30 genera: *Salmonella*, *Shigella*, *Chlamydia*, *Helicobacter*, *Yersinia*, *Bordatella*, *Pseudomonas*, *Neisseria*, *Vibrio*, *Haemophilus*, *Mycoplasma*, *Streptomyces*, *Treponema*, *Coxiella*,

Ehrlichia, Brucella, Streptobacillus, Fusospirocheta, Spirillum, Ureaplasma, Spirochaeta, Mycoplasma, Actinomycetes, Borrelia, Bacteroides, Trichomonas, Branhamella, Pasteurella, Clostridium, Corynebacterium, Listeria, Bacillus, Erysipelothrix, Rhodococcus,
5 *Escherichia, Klebsiella, Pseudomonas, Enterobacter, Serratia, Staphylococcus, Streptococcus, Legionella, Mycobacterium, Proteus, Campylobacter, Enterococcus, Acinetobacter, Morganella, Moraxella, Citrobacter, Rickettsia, Rochlimaeae, as well as bacterial species such as: P. aeruginosa; E. coli, P. cepacia, S. epidermis, E. faecalis, S. pneumoniae, S. aureus, N. meningitidis, S. pyogenes, Pasteurella multocida, Treponema*
10 *pallidum, and P. mirabilis.*

Examples of heterologous gene sequences derived from pathogenic fungi, include, but are not limited to, antigens derived from fungi such as *Cryptococcus neoformans; Blastomyces dermatitidis; Aiellomyces dermatitidis; Histoplasma capsulatum; Coccidioides immitis; Candida species, including C. albicans, C. tropicalis, C. parapsilosis, C.*
15 *guilliermondii and C. krusei, Aspergillus species, including A. fumigatus, A. flavus and A. niger, Rhizopus species; Rhizomucor species; Cunninghamhamella species; Apophysomyces species, including A. saksenaea, A. mucor and A. absidia; Sporothrix schenckii, Paracoccidioides brasiliensis; Pseudallescheria boydii, Torulopsis glabrata; Trichophyton species, Microsporum species and Dermatophyres species, as well as any other yeast or*
20 *fungus now known or later identified to be pathogenic.*

Finally, examples of heterologous gene sequences derived from parasites include, but are not limited to, antigens derived from members of the Apicomplexa phylum such as, for example, *Babesia, Toxoplasma, Plasmodium, Eimeria, Isospora, Atoxoplasma, Cystoisospora, Hammondia, Besnitiella, Sarcocystis, Frenkelia, Haemoproteus,*
25 *Leucocytozoon, Theileria, Perkinsus and Gregarina spp.; Pneumocystis carinii;* members of the Microspora phylum such as, for example, *Nosema, Enterocytozoon, Encephalitozoon, Septata, Mrazekia, Amblyospora, Ameson, Glugea, Pleistophora and Microsporidium spp.;* and members of the Ascetosporea phylum such as, for example, *Haplosporidium spp., as well as species including Plasmodium falciparum, P. vivax, P. ovale, P. malaria;*
30 *Toxoplasma gondii; Leishmania mexicana, L. tropica, L. major, L. aethiopica, L. donovani, Trypanosoma cruzi, T. brucei, Schistosoma mansoni, S. haematobium, S. japonium;*

Trichinella spiralis; *Wuchereria bancrofti*; *Brugia malayi*; *Entamoeba histolytica*;
Enterobius vermicularis; *Taenia solium*, *T. saginata*, *Trichomonas vaginalis*, *T. hominis*,
T. tenax; *Giardia lamblia*; *Cryptosporidium parvum*; *Pneumocystis carinii*, *Babesia bovis*, *B.*
5 *divergens*, *B. microti*, *Isospora belli*, *L. hominis*; *Dientamoeba fragilis*; *Onchocerca*
volvulus; *Ascaris lumbricoides*; *Necator americanis*; *Ancylostoma duodenale*;
Strongyloides stercoralis; *Capillaria philippinensis*; *Angiostrongylus cantonensis*;
Hymenolepis nana; *Diphyllobothrium latum*; *Echinococcus granulosus*, *E. multilocularis*;
10 *Paragonimus westermani*, *P. caliensis*; *Chlonorchis sinensis*; *Opisthorchis felinae*, *G.*
Viverini, *Fasciola hepatica*, *Sarcoptes scabiei*, *Pediculus humanus*; *Phthirus pubis*; and
Dermatobia hominis, as well as any other parasite now known or later identified to be
pathogenic.

One approach for constructing these hybrid molecules is to insert the heterologous
coding sequence into a DNA complement of a PIV gene so that the heterologous sequence is
15 flanked by the viral sequences required for viral polymerase activity; i.e., the viral
polymerase binding site/promoter, hereinafter referred to as the viral polymerase binding
site, and a polyadenylation site. In a preferred embodiment, the heterologous coding
sequence is flanked by the viral sequences that comprise the replication promoters of the 5'
and 3' termini, the gene start and gene end sequences, and the packaging signals that are
20 found in the 5' and/or the 3' termini. In an alternative approach, oligonucleotides encoding
the viral polymerase binding site, e.g., the complement of the 3'-terminus or both termini of
the virus genomic segment can be ligated to the heterologous coding sequence to construct
the hybrid molecule. The placement of a foreign gene or segment of a foreign gene within a
target sequence was formerly dictated by the presence of appropriate restriction enzyme
25 sites within the target sequence. However, recent advances in molecular biology have
lessened this problem greatly. Restriction enzyme sites can readily be placed anywhere
within a target sequence through the use of site-directed mutagenesis (e.g., see, for example,
the techniques described by Kunkel, 1985, Proc. Natl. Acad. Sci. U.S.A. 82;488).
Variations in polymerase chain reaction (PCR) technology, described infra, also allow for
30 the specific insertion of sequences (i.e., restriction enzyme sites) and allow for the facile
construction of hybrid molecules. Alternatively, PCR reactions could be used to prepare

recombinant templates without the need of cloning. For example, PCR reactions could be used to prepare double-stranded DNA molecules containing a DNA-directed RNA polymerase promoter (e.g., bacteriophage T3, T7 or SP6) and the hybrid sequence containing the heterologous gene and the PIV polymerase binding site. RNA templates could then be transcribed directly from this recombinant DNA. In yet another embodiment, the recombinant RNA templates may be prepared by ligating RNAs specifying the negative polarity of the heterologous gene and the viral polymerase binding site using an RNA ligase.

In addition, one or more nucleotides can be added at the 3' end of the HN gene in the untranslated region to adhere to the "Rule of Six" which may be important in obtaining virus rescue. The "Rule of Six" applies to many paramyxoviruses and states that the RNA nucleotide genome must be divisible by six to be functional. The addition of nucleotides can be accomplished by techniques known in the art such as using a commercial mutagenesis kits such as the QuikChange mutagenesis kit (Stratagene). After addition of the appropriate number of nucleotides, the correct hPIV3 F and HN gene DNA fragment can then be isolated by digestion with appropriate restriction enzyme and gel purification. Sequence requirements for viral polymerase activity and constructs which may be used in accordance with the invention are described in the subsections below.

5.1.1. INSERTION OF THE HETEROLOGOUS GENE SEQUENCE

Insertion of a foreign gene sequence into the nonsegmented genome of PIV can be accomplished by either a complete replacement of a viral coding region with a foreign gene or by a partial replacement. Complete replacement would probably best be accomplished through the use of PCR-directed mutagenesis. Briefly, PCR-primer A would contain, from the 5' to 3' end: a unique restriction enzyme site, such as a class IIS restriction enzyme site (i.e., a "shifter" enzyme; that recognizes a specific sequence but cleaves the DNA either upstream or downstream of that sequence); a stretch of nucleotides complementary to a region of the PIV gene; and a stretch of nucleotides complementary to the carboxy-terminus coding portion of the foreign gene product. PCR-primer B would contain from the 5' to 3' end: a unique restriction enzyme site; a stretch of nucleotides complementary to a PIV gene; and a stretch of nucleotides corresponding to the 5' coding portion of the foreign gene.

After a PCR reaction using these primers with a cloned copy of the foreign gene, the product may be excised and cloned using the unique restriction sites. Digestion with the class IIS enzyme and transcription with the purified phage polymerase would generate an RNA molecule containing the exact untranslated ends of the PIV gene with a foreign gene insertion. In an alternate embodiment, PCR-primed reactions could be used to prepare double-stranded DNA containing the bacteriophage promoter sequence, and the hybrid gene sequence so that RNA templates can be transcribed directly without cloning.

5.1.2. INSERTION OF THE HETEROLOGOUS GENE SEQUENCE INTO THE HN GENE

The hemagglutinin and neuraminidase activities of PIV are coded for by a single gene, HN. The HN protein is a major surface glycoprotein of the virus. For a variety of viruses, such as parainfluenza, the hemagglutinin and neuraminidase proteins have been demonstrated to contain a number of antigenic sites. Consequently, this protein is a potential target for the humoral immune response after infection. Therefore, substitution of antigenic sites within HN with a portion of a foreign protein may provide for a vigorous humoral response against this foreign peptide. If a sequence is inserted within the HN molecule and it is expressed on the outside surface of the HN it will be immunogenic. For example, a peptide derived from gp160 of HIV could replace an antigenic site of the HN protein, resulting in the elicitation of both a humoral immune response. In a different approach, the foreign peptide sequence may be inserted within the antigenic site without deleting any viral sequences. Expression products of such constructs may be useful in vaccines against the foreign antigen, and may indeed circumvent a problem discussed earlier, that of propagation of the recombinant virus in the vaccinated host. An intact HN molecule with a substitution only in antigenic sites may allow for HN function and thus allow for the construction of a viable virus. Therefore, this virus can be grown without the need for additional helper functions. The virus may also be attenuated in other ways to avoid any danger of accidental escape.

Other hybrid constructions may be made to express proteins on the cell surface or enable them to be released from the cell. As a surface glycoprotein, the HN has an amino-terminal cleavable signal sequence necessary for transport to the cell surface, and a carboxy-

terminal sequence necessary for membrane anchoring. In order to express an intact foreign protein on the cell surface it may be necessary to use these HN signals to create a hybrid protein. In this case, the fusion protein may be expressed as a separate fusion protein from an additional internal promoter. Alternatively, if only the transport signals are present and the membrane anchoring domain is absent, the protein may be secreted out of the cell.

5.1.3. CONSTRUCTION OF BICISTRONIC RNA

Bicistronic mRNA could be constructed to permit internal initiation of translation of viral sequences and allow for the expression of foreign protein coding sequences from the regular terminal initiation site. Alternatively, a bicistronic mRNA sequence may be constructed wherein the viral sequence is translated from the regular terminal open reading frame, while the foreign sequence is initiated from an internal site. Certain internal ribosome entry site (IRES) sequences may be utilized. The IRES sequences which are chosen should be short enough to not interfere with parainfluenza packaging limitations. Thus, it is preferable that the IRES chosen for such a bicistronic approach be no more than 500 nucleotides in length, with less than 250 nucleotides being preferred. Further, it is preferable that the IRES utilized not share sequence or structural homology with picornaviral elements. Preferred IRES elements include, but are not limited to the mammalian BiP IRES and the hepatitis C virus IRES.

Alternatively, a foreign protein may be expressed from a new internal transcriptional unit in which the transcriptional unit has an initiation site and polyadenylation site. In another embodiment, the foreign gene is inserted into a PIV gene such that the resulting expressed protein is a fusion protein.

5.2. EXPRESSION OF HETEROLOGOUS GENE PRODUCTS USING RECOMBINANT cDNA AND RNA TEMPLATES

The recombinant templates prepared as described above can be used in a variety of ways to express the heterologous gene products in appropriate host cells or to create chimeric viruses that express the heterologous gene products. In one embodiment, the recombinant cDNA can be used to transfect appropriate host cells and the resulting RNA may direct the expression of the heterologous gene product at high levels. Host cell systems

which provide for high levels of expression include continuous cell lines that supply viral functions such as cell lines superinfected with PIV, cell lines engineered to complement PIV functions, etc.

5 In an alternate embodiment of the invention, the recombinant templates may be used to transfect cell lines that express a viral polymerase protein in order to achieve expression of the heterologous gene product. To this end, transformed cell lines that express a polymerase protein such as the L protein may be utilized as appropriate host cells. Host
10 cells may be similarly engineered to provide other viral functions or additional functions such as HN, NP or N.

In another embodiment, a helper virus may provide the RNA polymerase protein utilized by the cells in order to achieve expression of the heterologous gene product. In yet another embodiment, cells may be transfected with vectors encoding viral proteins such as the N or NP, P and L proteins.

15 **5.3. RESCUE OF RECOMBINANT VIRUS PARTICLES**

In order to prepare chimeric virus, modified cDNAs, virus RNAs, or RNA coding for the PIV genome and/or foreign proteins in the plus or minus sense may be used to transfect cells which provide viral proteins and functions required for replication and rescue. Alternatively, cells may be transfected with helper virus before, during, or after transfection
20 by the DNA or RNA molecule coding for the PIV genome and/or foreign proteins. The synthetic recombinant plasmid PIV DNAs and RNAs can be replicated and rescued into infectious virus particles by any number of techniques known in the art, as described in U.S. Patent No. 5,166,057 issued November 24, 1992; in U.S. Patent No. 5,854,037 issued
25 December 29, 1998; in European Patent Publication EP 0702085A1, published February 20, 1996; in U.S. Patent Application Serial No. 09/152,845; in International Patent Publications PCT WO97/12032 published April 3, 1997; WO96/34625 published November 7, 1996; in
European Patent Publication EP-A780475; WO 99/02657 published January 21, 1999; WO 98/53078 published November 26, 1998; WO 98/02530 published January 22, 1998; WO
30 99/15672 published April 1, 1999; WO 98/13501 published April 2, 1998; WO 97/06270 published February 20, 1997; and EPO 780 47SA1 published June 25, 1997, each of which is incorporated by reference herein in its entirety.

In one embodiment, of the present invention, synthetic recombinant viral RNAs may be prepared that contain the non-coding regions of the negative strand virus RNA which are essential for the recognition by viral polymerases and for packaging signals
5 necessary to generate a mature virion. There are a number of different approaches which may be used to apply the reverse genetics approach to rescue negative strand RNA viruses. First, the recombinant RNAs are synthesized from a recombinant DNA template and reconstituted in vitro with purified viral polymerase complex to form recombinant
10 ribonucleoproteins (RNPs) which can be used to transfect cells. In another approach, a more efficient transfection is achieved if the viral polymerase proteins are present during transcription of the synthetic RNAs either in vitro or in vivo. With this approach the synthetic RNAs may be transcribed from cDNA plasmids which are either co-transcribed in vitro with cDNA plasmids encoding the polymerase proteins, or transcribed in vivo in the presence of polymerase proteins, i.e., in cells which transiently or constitutively express the
15 polymerase proteins.

In additional approaches described herein, the production of infectious chimeric virus may be replicated in host cell systems that express a PIV viral polymerase protein (e.g., in virus/host cell expression systems; transformed cell lines engineered to express a polymerase protein, etc.), so that infectious chimeric virus are rescued. In this instance,
20 helper virus need not be utilized since this function is provided by the viral polymerase proteins expressed.

In accordance with the present invention, any technique known to those of skill in the art may be used to achieve replication and rescue of chimeric viruses. One approach involves supplying viral proteins and functions required for replication in vitro prior to
25 transfecting host cells. In such an embodiment, viral proteins may be supplied in the form of wildtype virus, helper virus, purified viral proteins or recombinantly expressed viral proteins. The viral proteins may be supplied prior to, during or post transcription of the synthetic cDNAs or RNAs encoding the chimeric virus. The entire mixture may be used to transfect host cells. In another approach, viral proteins and functions required for
30 replication may be supplied prior to or during transcription of the synthetic cDNAs or RNAs encoding the chimeric virus. In such an embodiment, viral proteins and functions required

for replication are supplied in the form of wildtype virus, helper virus, viral extracts, synthetic cDNAs or RNAs which express the viral proteins are introduced into the host cell via infection or transfection. This infection/transfection takes place prior to or simultaneous
5 to the introduction of the synthetic cDNAs or RNAs encoding the chimeric virus.

In a particularly desirable approach, cells engineered to express all PIV viral genes may result in the production of infectious chimeric virus which contain the desired genotype; thus eliminating the need for a selection system. Theoretically, one can replace any one of the six genes or part of any one of the six genes encoding structural proteins of
10 PIV with a foreign sequence. However, a necessary part of this equation is the ability to propagate the defective virus (defective because a normal viral gene product is missing or altered). A number of possible approaches exist to circumvent this problem. In one approach a virus having a mutant protein can be grown in cell lines which are constructed to constitutively express the wild type version of the same protein. By this way, the cell line
15 complements the mutation in the virus. Similar techniques may be used to construct transformed cell lines that constitutively express any of the PIV genes. These cell lines which are made to express the viral protein may be used to complement the defect in the recombinant virus and thereby propagate it. Alternatively, certain natural host range systems may be available to propagate recombinant virus.

In yet another embodiment, viral proteins and functions required for replication may be supplied as genetic material in the form of synthetic cDNAs or RNAs so that they are co-transcribed with the synthetic cDNAs or RNAs encoding the chimeric virus. In a particularly desirable approach, plasmids which express the chimeric virus and the viral
20 polymerase and/or other viral functions are co-transfected into host cells. For example, plasmids encoding the genomic or antigenomic PIV RNA, either wild type or modified, may
25 be co-transfected into host cells with plasmids encoding the PIV viral polymerase proteins NP or N, P or L. Alternatively, rescue of chimeric PIV3/PIV3 virus may be accomplished by the use of Modified Vaccinia Virus Ankara (MVA), or a combination of MVA and plasmids encoding the polymerase proteins. For example, MVA can be transfected into
30 HeLa or Vero cells. After infection with MVA, a full length antigenomic bPIV3/hPIV3 cDNA may be transfected into the HeLa or Vero cells together with the NP, P, and L

encoding expression plasmids. The cells and cell supernatant can subsequently be harvested and subjected to a single freeze-thaw cycle. The resulting cell lysate may then be used to infect a fresh HeLa or Vero cell monolayer in the presence of 1-beta-D-
5 arabinofuranosylcytosine (ara C), a replication inhibitor of vaccinia virus, to generate a virus stock. The supernatant and cells from these plates can then be harvested, freeze-thawed once and the presence of bPIV3 virus particles assayed for by immunostaining of virus plaques using PIV3-specific antiserum.

10 Another approach to propagating the recombinant virus may involve co-cultivation with wild-type virus. This could be done by simply taking recombinant virus and co-infecting cells with this and another wild-type virus (preferably a vaccine strain). The wild-type virus should complement for the defective virus gene product and allow growth of both the wild-type and recombinant virus. Alternatively, a helper virus may be used to support
15 propagation of the recombinant virus.

15 In another approach, synthetic templates may be replicated in cells co-infected with recombinant viruses that express the PIV virus polymerase protein. In fact, this method may be used to rescue recombinant infectious virus in accordance with the invention. To this end, the PIV polymerase protein may be expressed in any expression vector/host cell system, including but not limited to viral expression vectors (e.g., vaccinia virus,
20 adenovirus, baculovirus, etc.) or cell lines that express a polymerase protein (e.g., see Krystal et al., 1986, Proc. Natl. Acad. Sci. USA 83: 2709-2713). Moreover, infection of host cells expressing all six PIV proteins may result in the production of infectious chimeric virus particles. This system would eliminate the need for a selection system, as all
25 recombinant virus produced would be of the desired genotype. It should be noted that it may be possible to construct a recombinant virus without altering virus viability. These altered viruses would then be growth competent and would not need helper functions to replicate.

30

5.4. VACCINE FORMULATIONS USING THE CHIMERIC VIRUSES

The invention encompasses vaccine formulations comprising the engineered
5 negative strand RNA virus of the present invention. The recombinant PIV viruses of the present invention may be used as a vehicle to express foreign epitopes that induce a protective response to any of a variety of pathogens. In a specific embodiment, the invention encompasses the use of recombinant bPIV viruses which have been modified in vaccine formulations to confer protection against hPIV infection.

10 The invention encompasses vaccine formulations to be administered to humans and animals which are useful to protect against PIV, influenza, RSV, Sendai virus, mumps, laryngotracheitis virus, simianvirus 5, human papillomavirus, as well as other viruses and pathogens.

In one embodiment, the invention encompasses vaccine formulations which are
15 useful against domestic animal disease causing agents including rabies virus, feline leukemia virus (FLV) and canine distemper virus. In yet another embodiment, the invention encompasses vaccine formulations which are useful to protect livestock against vesicular stomatitis virus, rabies virus, rinderpest virus, swinepox virus, and further, to protect wild animals against rabies virus.

20 Attenuated viruses generated by the reverse genetics approach can be used in the vaccine and pharmaceutical formulations described herein. Reverse genetics techniques can also be used to engineer additional mutations to other viral genes important for vaccine production -- i.e., the epitopes of useful vaccine strain variants can be engineered into the attenuated virus. Alternatively, completely foreign epitopes, including antigens derived
25 from other viral or non-viral pathogens can be engineered into the attenuated strain. For example, antigens of non-related viruses such as HIV (gp160, gp120, gp41) parasite antigens (e.g., malaria), bacterial or fungal antigens or tumor antigens can be engineered into the attenuated strain. Alternatively, epitopes which alter the tropism of the virus in vivo can be engineered into the chimeric attenuated viruses of the invention.

30 Virtually any heterologous gene sequence may be constructed into the chimeric viruses of the invention for use in vaccines. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing

antibodies may be expressed by or as part of the chimeric viruses. For example, heterologous gene sequences that can be constructed into the chimeric viruses of the invention include, but are not limited to influenza and parainfluenza hemagglutinin
5 neuraminidase and fusion glycoproteins such as the HN and F genes of human PIV3. In yet another embodiment, heterologous gene sequences that can be engineered into the chimeric viruses include those that encode proteins with immunopotentiating activities. Examples of immunopotentiating proteins include, but are not limited to, cytokines, interferon type 1, gamma interferon, colony stimulating factors, interleukin -1, -2, -4, -5, -6, -12.

10 In addition, heterologous gene sequences that can be constructed into the chimeric viruses of the invention for use in vaccines include but are not limited to sequences derived from a human immunodeficiency virus (HIV), preferably type 1 or type 2. In a preferred embodiment, an immunogenic HIV-derived peptide which may be the source of an antigen
15 may be constructed into a chimeric PIV that may then be used to elicit a vertebrate immune response. Such HIV-derived peptides may include, but are not limited to sequences derived from the env gene (i.e., sequences encoding all or part of gp160, gp120, and/or gp41), the pol gene (i.e., sequences encoding all or part of reverse transcriptase, endonuclease, protease, and/or integrase), the gag gene (i.e., sequences encoding all or part of p7, p6, p55, p17/18, p24/25), tat, rev, nef, vif, vpu, vpr, and/or vpx.

20 Other heterologous sequences may be derived from hepatitis B virus surface antigen (HBsAg); hepatitis A or C virus surface antigens, the glycoproteins of Epstein Barr virus; the glycoproteins of human papillomavirus; the glycoproteins of respiratory syncytial virus, parainfluenza virus, Sendai virus, simianvirus 5 or mumps virus; the glycoproteins of
25 influenza virus; the glycoproteins of herpes virus (e.g. gD, gE); VP1 of poliovirus; antigenic determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of anti-idiotypic immunoglobulins that mimic such epitopes may be constructed into the chimeric viruses of the invention.

30 Other heterologous sequences may be derived from tumor antigens, and the resulting chimeric viruses be used to generate an immune response against the tumor cells leading to tumor regression in vivo. These vaccines may be used in combination with other

therapeutic regimens, including but not limited to chemotherapy, radiation therapy, surgery, bone marrow transplantation, etc. for the treatment of tumors. In accordance with the present invention, recombinant viruses may be engineered to express tumor-associated antigens (TAAs), including but not limited to, human tumor antigens recognized by T cells (Robbins and Kawakami, 1996, Curr. Opin. Immunol. 8:628-636, incorporated herein by reference in its entirety), melanocyte lineage proteins, including gp100, MART-1/MelanA, TRP-1 (gp75), tyrosinase; Tumor-specific widely shared antigens, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-1, N-acetylglucosaminyltransferase-V, p15; Tumor-specific mutated antigens, β -catenin, MUM-1, CDK4; Nonmelanoma antigens for breast, ovarian, cervical and pancreatic carcinoma, HER-2/neu, human papillomavirus -E6, -E7, MUC-1.

Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification. Additionally, as bPIV has been demonstrated to be non-pathogenic in humans, this virus is highly suited for use as a live vaccine.

In this regard, the use of genetically engineered PIV (vectors) for vaccine purposes may desire the presence of attenuation characteristics in these strains. The introduction of appropriate mutations (e.g., deletions) into the templates used for transfection may provide the novel viruses with attenuation characteristics. For example, specific missense mutations which are associated with temperature sensitivity or cold adaption can be made into deletion mutations. These mutations should be more stable than the point mutations associated with cold or temperature sensitive mutants and reversion frequencies should be extremely low.

Alternatively, chimeric viruses with "suicide" characteristics may be constructed. Such viruses would go through only one or a few rounds of replication within the host. When used as a vaccine, the recombinant virus would go through limited replication cycle(s) and induce a sufficient level of immune response but it would not go further in the human host and cause disease. Recombinant viruses lacking one or more of the PIV genes

or possessing mutated PIV genes would not be able to undergo successive rounds of replication. Defective viruses can be produced in cell lines which permanently express such a gene(s). Viruses lacking an essential gene(s) will be replicated in these cell lines but when administered to the human host will not be able to complete a round of replication. Such preparations may transcribe and translate --in this abortive cycle -- a sufficient number of genes to induce an immune response. Alternatively, larger quantities of the strains could be administered, so that these preparations serve as inactivated (killed) virus vaccines. For inactivated vaccines, it is preferred that the heterologous gene product be expressed as a viral component, so that the gene product is associated with the virion. The advantage of such preparations is that they contain native proteins and do not undergo inactivation by treatment with formalin or other agents used in the manufacturing of killed virus vaccines. Alternatively, mutated PIV made from cDNA may be highly attenuated so that it replicates for only a few rounds.

In another embodiment of this aspect of the invention, inactivated vaccine formulations may be prepared using conventional techniques to "kill" the chimeric viruses. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity of the virus is destroyed without affecting its immunogenicity. In order to prepare inactivated vaccines, the chimeric virus may be grown in cell culture or in the allantois of the chick embryo, purified by zonal ultracentrifugation, inactivated by formaldehyde or β -propiolactone, and pooled. The resulting vaccine is usually inoculated intramuscularly.

Inactivated viruses may be formulated with a suitable adjuvant in order to enhance the immunological response. Such adjuvants may include but are not limited to mineral gels, e.g., aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols, polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG and *Corynebacterium parvum*.

Many methods may be used to introduce the vaccine formulations described above, these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be preferable to introduce the

chimeric virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed.

5

6. **EXAMPLE 1: CONSTRUCTION AND CLONING OF
CHIMERIC BOVINE PARAINFLUENZA 3 /
HUMAN PARAINFLUENZA 3 cDNA**

For the purpose of interchanging the F and HN genes of bPIV3 with those of hPIV3, additional restriction enzyme sites were introduced into the infectious bPIV3 cDNA. Using
10 site-directed mutagenesis, a unique Nhe 1 site was introduced at nucleotide position 5041 and a Sal 1 site was introduced at nt 8529 in the bPIV3 cDNA. The modified full-length bPIV3 cDNA was treated with Nhe 1 and Sal 1 and a ~14 kb DNA fragment encompassing all of the viral bPIV3 sequences except the F and HN genes, was isolated by gel purification.

15 To obtain the hPIV3 F and HN gene sequences, a 10 cm dish of confluent Vero cells was infected with hPIV3/Tex/12084/1983. After 3 days of incubation at 37°C, the cells were harvested and total RNA was isolated using RNA STAT-LS 50 (Tel-Test Inc.). Viral cDNA was generated by reverse transcription using a hPIV3 specific oligo annealing at
20 position 4828 of the hPIV3 genome. The hPIV3 F and HN genes were amplified by PCR (polymerase chain reaction) using Taq polymerase. The PCR product was cloned into the pT/A TOPO cloning vector (Invitrogen) and from two clones (#11 and #14) the hPIV3 F and HN genes were sequenced. In the sequence revealed for clone #11, the F gene was correct, but the HN gene contained aberrant sequences. While for the second clone #14, the HN gene was correct, but the F gene contained aberrant stop codons. Thus, a functional
25 hPIV3 F and HN genes-containing plasmid was constructed by combining the correct F gene of #11 with the correct HN gene of #14 in the following manner. Both hPIV3 plasmids (#11 and #14) were digested with Nhe1 and EcoR1. A 1.6 kb fragment harboring the correct F gene was isolated from clone #11 and a 8.5 kb fragment containing the correct HN gene and plasmid sequences, was isolated from clone #14. The two fragments were
30 ligated to produce the intact hPIV3 F and HN genes-containing plasmid. The correct sequence was confirmed by DNA sequence analysis. Finally, a single nucleotide was added at the 3' end of the HN gene in the untranslated region to adhere to the "Rule of Six." The

addition of the single nucleotide was accomplished by using the QuikChange mutagenesis kit (Stratagene) and was confirmed by DNA sequencing. The correct hPIV3 F and HN gene DNA fragment was then isolated by digestion with Nhe 1 and Sal 1 and a 3.5 kb DNA fragment was gel purified.

The full-length bPIV3/hPIV3 chimeric cDNA was constructed by ligating the 14.5 kb DNA fragment harboring bPIV3 sequences described above and the 3.5 kb DNA fragment containing the hPIV3 F and HN genes (see Fig. 1). The full-length chimeric plasmid DNA was confirmed by extensive restriction enzyme mapping. In addition, the M/F and HN/L gene junctions of the chimeric construct were confirmed by DNA sequencing to both contain bPIV3 and hPIV3 sequences as well as a Nhe 1 and a Sal 1 restriction enzyme site, respectively.

7. EXAMPLE 2: RESCUE OF CHIMERIC BOVINE PARAINFLUENZA TYPE 3 / HUMAN PARAINFLUENZA TYPE 3 VIRUS IN HELA CELLS AND VERO CELLS

Rescue of the chimeric bPIV3/hPIV3 virus was done using a similar procedure as for bPIV3 rescue. Rescue of bPIV3/hPIV3 chimeric virus by reverse genetics was carried out in HeLa cells using LipofecTACE (Gibco/BRL). The 80% confluent HeLa cells or Vero cells were infected with MVA at an MOI of 4. One hour post-infection, the full-length anti-genomic bPIV3/hPIV3 cDNA (4 µg) was transfected into the infected HeLa or Vero cells together with the NP (0.4 µg), P (0.4 µg), and L/pCITE (0.2 µg) expression plasmids. Forty hours post-transfection, the cells and the cell supernatant were harvested (P0) and subjected to a single freeze-thaw cycle. The resulting cell lysate was then used to infect a fresh Vero cell monolayer in the presence of 1-beta-D-arabinofuranosylcytosine (ara C), a replication inhibitor of vaccinia virus, to generate a P1 virus stock. The supernatant and cells from these plates were harvested, freeze-thawed once and the presence of bPIV3 virus particles was assayed for by immunostaining of virus plaques using PIV3-specific antiserum. The cell lysates of the P1 harvest resulted in complete CPE of the Vero cell monolayers and immunostaining indicated the presence of an extensive virus infection.

8. **EXAMPLE 3: CONFIRMATION OF CHIMERIC BOVINE
PARAINFLUENZA TYPE 3 / HUMAN PARAINFLUENZA
TYPE 3 VIRUS RESCUE BY RT-PCR**

5

To ascertain that the rescued virus is chimeric in nature, i.e. the virus contains hPIV3 F and HN gene sequences in a bPIV3 backbone, the viral RNA genome was analyzed further by RT-PCR. Vero cells, infected with the P1 virus stock of three independently derived isolates of bPIV3/hPIV3 were harvested and total RNA was isolated. The viral
10 RNA was amplified using an oligo that anneals at position 4757 of bPIV3. A viral region from nt 5255 to 6255 was amplified by PCR. The resulting 1 kb PCR fragment should contain hPIV3 sequences. This was confirmed by digestion with enzymes (SacI and Bgl II) specific for hPIV3 and that do not cut in the complementary region of bPIV3 (see Fig. 1). As expected, SacI and Bgl II cut the PCR fragment into smaller fragments confirming that
15 the isolated sequences are derived from hPIV3 (see lanes 3, 5, 7). In addition, a region in the polymerase L gene from nt 9075 to nt 10469 was amplified by PCR. This region should contain bPIV3 sequences. Again the resulting 1.4 kb PCR fragment was digested using enzyme specific for bPIV3 (PvuII and BamHI) that do not cut in the equivalent region of hPIV3 (Fig. 2). The 1.4 kb fragment was indeed digested by both PvuII and BamHI
20 confirming that the polymerase gene is bPIV3 in origin (see lanes 3, 4, 6, 7, 9 and 10 of Fig. 2). In summary, the RT-PCR analysis shows that the rescued bPIV3/hPIV3 virus is chimeric in nature. It contains hPIV3 F and HN genes in a bPIV3 genetic backbone.

9. **EXAMPLE 4: THE CHIMERIC BOVINE PIV
DEMONSTRATE ATTENUATED PHENOTYPES AND ELICIT
STRONG PROTECTIVE RESPONSES WHEN
ADMINISTERED IN VIVO.**

25

Five week old Syrian Golden hamsters were infected with 5×10^5 pfu of wildtype bPIV3, recombinant bPIV3, hPIV3, human/bovine PIV3, and placebo. The five different
30 animal groups were kept separate in micro-isolator cages. Four days post-infection, the animals were sacrificed. The nasal turbinates and lungs of the animals were homogenized and stored at -80°C . Virus present in the tissues was determined by TCID₅₀ assays in

MDBK cells at 37°C. Virus infection was confirmed by hemabsorption with guinea pig red blood cells. Table 1 shows the replication titers of the different PIV3 strains in hamsters in the lungs and nasal turbinates. Note that recombinant bPIV3 and the bPIV3/hPIV3 chimeric viruses are attenuated in the lungs of the hamsters:

Table 1
Replication of PIV3 Viruses in Syrian
Golden Hamsters in the Nasal Turbinates and Lungs.

10	Virus Used to Infect the Animals	Nasal Turbinates Log ₁₀ TCID ₅₀ /g tissue	Lungs Log ₁₀ TCID ₅₀ /g tissue
	Wild type bPIV3	5.47 +/- 0.001	5.22 +/- 0.014
	Recombinant bPIV3	5.00 +/- 0.361	2.90 +/- 0.490
	Wild type hPIV3	4.86 +/- 0.072	4.81 +/- 0.123
15	BPIV3/h PIV3 chimeric virus	4.68 +/-0.104	3.73 +/- 0.418
	Placebo	0	0

20 Furthermore, serum samples collected from the hamsters prior to infection and at day 21 post-infection were analyzed in a hemagglutination inhibition assay. The serum samples were treated with receptor destroying enzyme (RDE, DENKA Seiken Co.) and non-specific agglutinins were removed by incubation with guinea pig red blood cells for 1 hour on ice. Wildtype bPIV3 and hPIV3 were added to two-fold serially diluted hamster serum samples. Finally, guinea pig red blood cells (0.5%) were added, and hemagglutination was allowed to occur at room temperature. Table 2 shows the antibody response generated in the hamsters upon being infected with the different PIV3 strains. Note that the bPIV3/hPIV3 chimeric virus generates as strong an antibody response against hPIV3 as does wild type hPIV3, far exceeding the response generated by the recombinant or wildtype bPIV3:

Table 2
Hemagglutination Inhibition Assay Using Serum from
Hamsters Infected with Different PIV3 Viruses.

5

Virus Used for Inoculation of the Hamsters	Hamster Serum Titers for	
	wt bPIV3	HPIV3
Recombinant bPIV3	1:16	1:16
Wt bPIV3	1:16	1:8
10 Wt hPIV3	1:4	1:128
bPIV3/hPIV3 chimeric virus	1:8	1:128
Placebo	<1:4	<1:4

These results demonstrate the properties of bPIV3/hPIV3 chimeric viruses of the present invention which make these recombinants suitable for use in vaccine formulations. Not only do the bPIV3/hPIV3 chimeric viruses demonstrate an attenuated phenotype when administered *in vivo*, but they also generate as strong an antibody response as the wildtype hPIV3. Thus, because the chimeric viruses of the present invention have a unique combination of having an attenuated phenotype and eliciting as strong an immune response as a wildtype hPIV, these chimeric viruses have the characteristics necessary for successful use in humans to inhibit and/or protect against infection with PIV.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any constructs, viruses or enzymes which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

- 5 1. A chimeric parainfluenza virus comprising a backbone encoded by nucleotide sequences derived from a Kansas-strain bovine parainfluenza virus type 3 genome in which Kansas-strain bPIV3 nucleotide sequences have been substituted with heterologous sequences or in which heterologous sequences have been added to the complete Kansas-strain bPIV3 genome.
- 10 2. The chimeric parainfluenza virus of claim 1, wherein the heterologous sequences are derived from a human parainfluenza virus.
- 15 3. The chimeric parainfluenza virus of claim 2, wherein the heterologous sequences encode the F and HN glycoproteins of a human parainfluenza virus.
- 20 4. The chimeric parainfluenza virus of claim 3, wherein the F and HN glycoproteins of an hPIV are derived from human parainfluenza virus type 3.
- 25 5. The chimeric parainfluenza virus of claim 1, wherein the heterologous sequences are derived from an influenza virus or from a respiratory syncytial virus.
6. The chimeric parainfluenza virus of claim 1, wherein the Kansas-strain bPIV3 backbone contains mutations or modifications, in addition to heterologous sequences, which result in a chimeric virus having a phenotype more suitable for use in vaccine formulations such as an attenuated phenotype or a phenotype with enhanced antigenicity.
- 30 7. A recombinant DNA or RNA molecule encoding a chimeric parainfluenza virus comprising nucleotide sequences derived from a Kansas-strain bovine parainfluenza virus type 3 genome in which Kansas-strain bPIV3 nucleotide sequences have been substituted with heterologous sequences or in which heterologous sequences have been added to the complete Kansas-strain bPIV3 genome.

8. The recombinant DNA or RNA molecule of claim 7, wherein the heterologous sequences are derived from a human parainfluenza virus.

5 9. The recombinant DNA or RNA molecule of claim 7, wherein the heterologous sequences are derived from an influenza virus.

10 10. The recombinant DNA or RNA molecule of claim 7, wherein the heterologous sequences are derived from a respiratory syncytial virus.

15 11. The recombinant DNA or RNA molecule of claim 7, wherein the nucleotide sequences derived from a Kansas-strain bovine parainfluenza virus type 3 genome contain mutations or modifications, in addition to heterologous sequences, which result in a chimeric virus having a phenotype more suitable for use in vaccine formulations such as an attenuated phenotype or a phenotype with enhanced antigenicity.

12. A vaccine formulation comprising a chimeric Kansas-strain bPIV3, the genome of which encodes a heterologous epitope, and a pharmaceutically acceptable excipient.

20 13. The vaccine formulation of claim 12, comprising genomic modifications or mutations which result in an attenuated phenotype or enhanced antigenicity.

25 14. The vaccine formulation of claim 13 in which the modification is derived from a naturally occurring mutant.

15. The vaccine formulation of claim 12 wherein the vaccine is used to modulate the immune response of humans, primates, horses, cows, sheep, pigs, goats, cats, avian species and rodents.

30 16. The vaccine formulation of claim 15, wherein the vaccine is used to modulate the immune response of human infants and children.

ABSTRACT

5 The present invention relates to recombinant bovine parainfluenza virus
(bPIV) cDNA or RNA which may be used to express heterologous gene products in
appropriate host cell systems and/or to rescue negative strand RNA recombinant viruses that
express, package, and/or present the heterologous gene product. The chimeric viruses and
expression products may advantageously be used in vaccine formulations including vaccines
10 against a broad range of pathogens and antigens.

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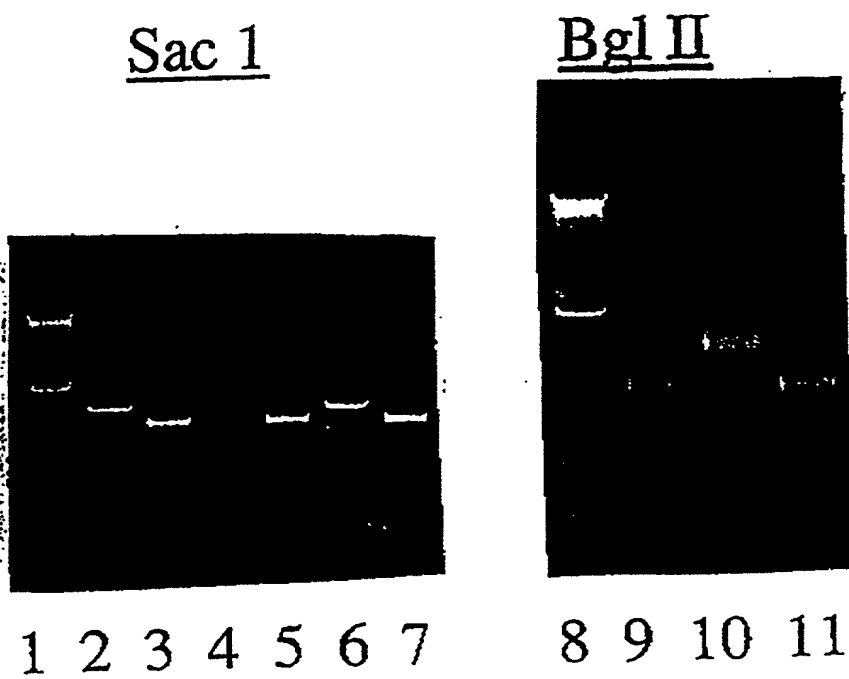


FIG. 1

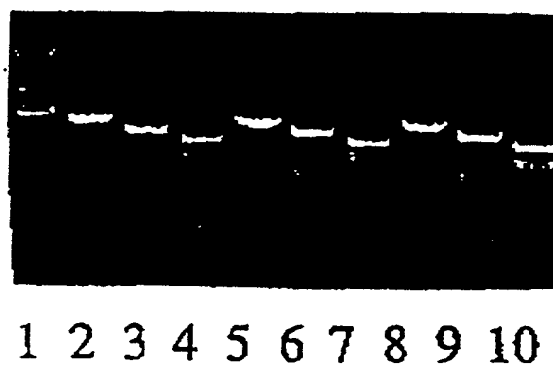


FIG. 2

DECLARATION AND POWER OF ATTORNEY

As a below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below at 201 et seq. underneath our respective names.

We believe we our the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

Recombinant Parainfluenza Virus Expression Systems and Vaccines

and for which a patent application:

☐ is attached hereto and includes amendment(s) filed on *(if applicable)*

☐ was filed in the United States on as Application No. *(for declaration not accompanying application)*

with amendment(s) filed on *(if applicable)*

☐ was filed as PCT international Application No. on and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE	DATE	DATE
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